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Lifespan differences between queens and workers are not explained by rates of molecular damage

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ABSTRACT

The biological processes that underlie senescence are of universal biological importance, yet they remain poorly understood. A popular theory proposes that senescence is the result of limited investment into mechanisms involved in the prevention and repair of molecular damage, leading to an accumulation of molecular damage with age. In ants, queen and worker lifespans differ by an order of magnitude, and this remarkable difference in lifespan has been shown to be associated with differences in the expression of genes involved in DNA and protein repair. Here we use the comet assay and Western Blotting for poly-ubiquitinated proteins to explore whether these differences in expression lead to differences in the accumulation of DNA damage (comet assay) or protein damage (protein ubiquitination) with age. Surprisingly, there was no difference between queens and workers in the rate of accumulation of DNA damage. We also found that levels of ubiquitinated proteins decreased with age, as previously reported in honeybees. This is in contrast to what has been found in model organisms such as worms and flies. Overall, these results reveal that the link between investment into macromolecular repair, age-related damage accumulation and lifespan is more complex than usually recognised.

1. Introduction

Senescence, that is the physiological decline in function that comes with age, is a universal and inescapable process for many sexual organisms, from yeast to humans (Gardner and Mangel, 1997). One frequently-evoked explanation for senescence proposes that naturally-occurring molecular damage accumulates with age, leading to a gradual breakdown of biological processes (Finkel and Holbrook, 2000). The Disposable Soma theory proposes that the rate of senescence can thus be moderated by increased investment into mechanisms that prevent or repair molecular damage (Kirkwood and Holliday, 1979), but that complete damage avoidance is rarely an optimal life-history strategy. Early research addressing this theory focused on anti-oxidant production as a means of damage prevention and has largely rejected the idea of a link with longevity, finding little association between investment into damage prevention and lifespan (Corona et al., 2005; Doonan et al., 2008; Gems and Doonan, 2009; Kenyon, 2010; Lucas and Keller, 2014; Pérez et al., 2009; Parker et al., 2004; Schneider et al., 2011; Wolschin and Amdam, 2007). However, less attention has been directed towards repair mechanisms (Guarente and Kenyon, 2000), despite evidence that failure of genes involved in DNA repair leads to accelerated ageing (Kenyon, 2005; Lombard et al., 2005).

The link between molecular repair and ageing has recently been investigated in the ant *Lasius niger* (Lucas et al., 2016), where queens can live over ten times longer than workers despite a lack of genetic differences between them. This study explored the expression of genes linked to DNA repair, and also to the Ubiquitin Proteasome System (UPS), a process whereby mis-folded or damaged proteins are labelled with ubiquitin for subsequent degradation, and which is associated with longevity in worms (Vilchez et al., 2012) and flies (Tonoki et al., 2009). The results revealed that the expression of genes involved in processes of DNA repair and the UPS was higher in queens compared to workers (Lucas et al., 2016). In order for these differences in gene expression to translate into differences in lifespan, they should result in differential accumulation of molecular damage between queens and workers. Here, we test the prediction that the age-related accumulation of DNA and protein damage is lower in queens compared to workers in *L. niger*. We quantified DNA damage by a comet assay and use Western Blotting to measure levels of ubiquitinated proteins, a proxy marker of damaged proteins which has been shown to accumulate with age in a range of species from worms to flies (Liu et al., 2011; Tonoki et al., 2009).

We measured DNA damage and protein ubiquitination in the heads and legs of queens and workers. The brain is critical for survival and function, and is therefore a tissue in which differing levels of somatic damage are likely to be most relevant to ageing. Legs represent a tissue

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whose function is the same between queens and workers and which should therefore suffer from little confounding variation. Many tissues serve different functions in queens and workers, and could therefore be exposed to qualitatively different biological processes. The choice of legs as a study tissue addresses this concern. While workers may exercise their legs more than queens, the biological role of this tissue does not change. In both legs and heads, we expected DNA damage and protein ubiquitination to increase with age, and to do so more quickly in workers than queens.

2. Methods

2.1. Sample collection

In *L. niger*, queens can live up to thirty years, while workers live for one year on average, to a maximum of three years (Hölldobler and Wilson, 1990; Kramer et al., 2016). Caste is determined during the developmental period in this species, and workers are thus unable to become queens even in the event of queen death. Workers in this species, regardless of rearing conditions, have to our knowledge never been reported to live longer than three years. Thus, if the rate of accumulation of molecular damage underlies the difference in lifespan between queens and workers, this should be apparent regardless of rearing conditions. In this study, we used 1-day-old and 1-year-old queens and workers to investigate whether the increase in DNA and protein damage over the first year of life is greater in workers than queens.

Queenless and queenright colonies were set up in the laboratory using ants collected from the campus of the University of Lausanne (UNIL). Colonies were maintained in controlled climate conditions (25 °C, 60% humidity) with a 12 h/12 h light/dark cycle and fed with 10% honey, mealworms, and an "ant diet" composed of agar, honey, eggs and a vitamin supplement (Bhatkar and Whitcomb, 1970 and Supplementary materials).

To set up queenright colonies, mated queens were collected just after mating flights in June and July, and transferred to a test tube half-filled with water, with a cotton plug between the queen and the water. These queens were allowed to raise their first brood of workers in the dark, using their own nutrient reserves, as they do naturally (Hölldobler and Wilson, 1990). After six weeks, the queens and their brood were transferred to plastic boxes coated in fluon and with holes in the lid. These boxes contained a plaster nest with an opaque lid in which the ants established their colony.

Queenright laboratory colonies are small and do not produce new queens. Since only field-raised queens can be obtained, we matched this condition by using field-raised workers. Queen and worker pupae were thus obtained from field colonies by taking queenless subsets of these colonies into the laboratory. To set up these queenless colonies, workers and brood were collected from established field colonies from May to June. These colonies contained around 100–500 workers, pupae and a small number of late-stage larvae.

To obtain 1-day-old queens and workers, newly emerged individuals were collected in 2014 from queenless colonies and flash frozen in liquid nitrogen. To obtain 1-year-old workers, we removed all adult workers from previously established queenright colonies in 2013 and added 40 worker pupae, or newly emerged workers, taken from queenless colonies. To ensure that no subsequent younger workers emerged, all new pupae were removed from these colonies each week. All workers were collected and flash-frozen one year later. Mean ± sd worker survival across the 10 colonies set up in this way was 0.62 ± 0.11 over the year of the experiment. Colony size at the start of the experiment was 40, since this is the number of workers we introduced.

To obtain 1-year-old queens, we established queenright colonies in 2013 by collecting newly-mated queens from the field. These colonies were then allowed to grow for six months. After this, in order to match any stress induced by the treatment that the colonies containing 1-year-old workers received, we removed all pupae each week and collected the queens when they were 1-year-old. Similarly, in order to broadly match the colony size of colonies containing 1-year-old workers, queenright colonies containing >40 workers were reduced to 40 workers by removal of a random subset of workers. Before worker removal, the mean number of workers per colony was 42.2. After removal, the mean number of workers per colony was 35.2. Of 14 queens that were allowed to set up colonies, 13 survived to one year of age, queen survival was thus 0.93.

In order to match the tissues in which the expression of DNA repair genes and protein ubiquitination genes have been studied (Lucas et al., 2016), we measured levels of DNA damage and protein ubiquitination in the heads and legs of *L. niger*.

An unavoidable outcome of our design is that workers collected as 1-year-old individuals may represent a biased subset of all workers, since they include only those that are able to survive this long. Unfortunately, this problem cannot be controlled experimentally since it is by definition impossible to obtain 1-year-old individuals that die before the end of their first year, or to know which 1-day-old individuals will survive for one year. Our experiment may therefore underestimate the rate of DNA damage accumulation in workers. Nevertheless, the remaining workers have still undergone a greater fraction of their expected lifespan than queens and thus we predicted that if an accumulation of DNA damage does underlie the difference in longevity between queens and workers, a lower rate of damage accumulation should be apparent in queens compared to workers that could be collected after one year.

2.2. Comet assay

To investigate the effects of age and caste on levels of DNA damage, we measured the extent of DNA strand breakage in 1-day and 1-year-old queens and workers using a comet assay (Singh et al., 1988), a technique that has been successfully used in many insect species (Augustyniak et al., 2016) and that is recognised as being very sensitive and require a relatively small amount of DNA (Dhawan et al., 2009). Cells from the head were obtained by cutting off the head and opening the chitin capsule with tweezers, followed by extraction of the tissue in a drop of PBS buffer, containing 20 mM EDTA and 10% DMSO (Park and Choi, 2007) and maceration with microsurgery forceps for 1 min. Macerated tissue was collected into a tube and 100 μL or 150 μL total PBS aliquot was used for worker or queen brain respectively to obtain a cell suspension. Legs were placed in a 10 μL drop of PBS, cut into small pieces with micro scissors, then placed in the same volume of PBS as was the brain tissue of dissected ant, and homogenised for 5 s in Minilys (Bertin Technologies, Montigny-le-Bretonneux, France) with five medium-sized beads in the tube.

The comet assay was performed under alkaline conditions according to Bilbao et al. (2002) with some minor modifications described in Augustyniak et al. (2006). Briefly, 60 μL of the cell suspension was mixed with 1.5% low melting point agarose (LMPA) (1:1, v/v) and placed onto slide previously covered with 1% normal melting point agarose (NMPA). Finally, 100 μL of 1.0% LMPA was spread over the cells and covered with cover slip. After solidification of the upper agarose layer in 4 °C and removal of cover slip the slide was placed in the lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 0.25 M NaOH, 1% TritonX-100 and 10% dimethyl sulfoxide (DMSO), pH 10.0), for 2 h at 4 °C for destruction of cell and nuclear membranes. Following washing the slide in $_{d}H_2O$, denaturation was performed in horizontal gel electrophoresis unit for 20 min in a buffer consisting of 300 mM NaOH and 0.9 mM Na2EDTA, pH > 13, and then electrophoresis was carried out for 20 min at 4 °C and 0.5 V/cm (0.3 A) electric field strength. The slides were neutralized (0.4 M Tris-HCl; pH 7.4), fixed with methanol, dried and stored at 4 °C until further analysis. The entire procedure was carried out in dimmed light. We obtained the following sample sizes; 1-day-old workers: twelve individuals from four colonies; 1-day-old queens: seven individuals from six colonies; 1-year-old workers: twelve individuals from four colonies; 1-year-old queens: six individuals from six colonies.

Before the comet analysis, slides were wetted for 5 min with $_dH_2O$, dyed with 0.1% DAPI and visualised under the fluorescence microscope equipped with a 355 nm excitation filter and 450 nm barrier filter. DNA damage was measured by Komet 5.5 (Kinetic Imaging, Liverpool, UK) image analysis system. At least 50 nuclei per slide were randomly selected and the percentage of DNA in the comet tail (TDNA) was measured.

The amount of material available was insufficient to include an analysis of the efficiency of DNA repair after exposure to damaging agent such as H_2O_2. Such experiments require a larger volume of the sample suspension for preparation of slides in several consecutive time intervals following damaging agent application. We consider that the fact that we were able to detect differences due to age provides a satisfactory indication that our analysis had the power to detect differences on biologically-relevant scales.

2.3. Detection of ubiquitinated proteins by Western Blot

As a proxy for protein damage, we measured levels of ubiquitinated proteins in 1-day-old and 1-year-old queens and workers by Western Blot. This was done by SDS-PAGE and immuno-blotting using a method similar to those described in Tonoki et al. (2009). Damaged proteins are labelled with poly-ubiquitin chains, and the levels of ubiquitinated proteins are therefore indicative of the amount of damaged proteins present in the sample. This method has previously been used effectively to investigate levels of protein ubiquitination in the context of ageing in *Drosophila* (Tonoki et al., 2009) and honeybees (Tolfsen et al., 2011).

Individuals were dissected on dry ice to isolate heads and legs, which were analysed separately in order to detect tissue-specific patters. One individual was used per queen replicate and 15 individuals were pooled for each worker replicate for both legs and heads. Pooling of workers was necessary to obtain sufficient material, while pooling of queens was not necessary due to their higher biomass. Proteins were extracted as follows: dissected tissues were crushed with a micropestle in extraction buffer (150 mM sodium chloride, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS, 5% 1 M Tris (pH 8.0), 1× protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO; ref.:P2714-1BTL)), then incubated at 4 °C for 30 min (briefly vortexed every 5 min) before being centrifuged for 10 min at 13,200 rpm and 4 °C. The supernatant was removed, transferred into a clean microcentrifuge tube and centrifuged for a further 10 min at 13,200 rpm and 4 °C. The supernatant was again removed, transferred into a clean microcentrifuge tube and stored at −80 °C. Four replicates were obtained for each age/caste/tissue combination. Samples from different tissues were then analysed separately.

Samples were separated on a 10% SDS-polyacrylamide gel. Each gel contained two samples from each age/caste combination of a given tissue. All gels were run in duplicates, with one gel stained with Coomassie Brilliant Blue to reveal total protein and the other used to transfer proteins onto a nitrocellulose membrane (0.2 μm). The membrane was blocked with a special blocking buffer for Fluorescent Western Blot (Rockland Immunochemicals, Limerick, PA; ref.:MB-070) and then probed with a mouse anti-ubiquitin primary antibody (Enzo Life Sciences, Farmingdale, NY; ref.: P4G7-H11, 1:1000 dilution). The blot was revealed using a goat anti-mouse secondary antibody AlexaFluor 680 (Invitrogen, ThermoFisher Scientific, Waltham, MA; ref.:A21058; 1:2000 dilution). The membrane and Coomassie-stained gel were then scanned on a LI-COR scanner and analysed by densitometry using the software *ImageJ*. The total protein detected on the Coomassie gel was used to normalise the amount of ubiquitinated proteins on the blots. As certain protein bands are closely associated with specific ages or castes, densitometry was not performed on the entire protein spectrum. Instead, for each tissue a region of the gel was chosen in which no caste- or age-specific bands were apparent. Images of all gels, and examples of gel image from each tissue after processing, are provided in Supplementary Figs. S1–3.

2.4. Statistical analysis

In order to control for the random effects of colony and individual id (comet assay) and gel (Western Blotting), data were analysed using general linear mixed models with normal errors and an identity link function, implemented with the *R* package *lme4*. Normality of residuals was tested using a Kolmogorov-Smirnov test (comet assay in heads: $n = 49$, $P = 0.31$; comet assay in legs: $n = 50$, $P = 0.81$; Western Blotting in heads: $n = 16$, $P = 0.86$; Western Blotting in legs: $n = 16$, $P = 0.58$) and homogeneity of variance was tested using a Bartlett test (comet assay in heads: $n = 49$, df = 3, $P = 0.21$; comet assay in legs: $n = 50$, df = 3, $P = 0.09$; Western Blotting in heads: $n = 16$, df = 3, $P = 0.86$; Western Blotting in legs: $n = 16$, df = 3, $P = 0.91$). Model comparison was performed using the *ANOVA* function in *R* with a χ^2 test of significance.

For the comet assay, the percentage of DNA present in the comet's tail was set as the dependent variable. Age ('1-day' or '1-year'), caste ('queen' or 'worker') and their interaction were set as categorical fixed effects, while colony of origin was set as a random effect. As two measures were obtained from each individual queen, individual id was also included in the model as a random effect. The effects of age and caste were investigated in heads and legs independently. To investigate the effect of tissue on DNA damage, we compared the model including the two random effects and tissue as a fixed effect with the null model including only the random effects. We also performed this comparison with the inclusion of age and caste as further fixed effects; the results were qualitatively the same.

For the Western Blotting, age, caste and their interaction were set as categorical fixed effects, while the gel of origin was set as a random effect. *P*-values for a given variable were obtained by comparing the model including that variable to the model in which that variable was removed. To test the effect of caste within an age group, models were built using data from a single age. The model with caste as a fixed effect and all random effects was compared to the model containing only random effects. To test the effect of age within a caste, models were built using data from a single caste. The model with age as a fixed effect and all random effects was compared to the model containing only random effects. Data from heads and legs were analysed separately. No comparison could be made of protein ubiquitination between heads and legs as these two tissues were run on different gels and thus the confounding effect of gel could not be controlled.

3. Results

3.1. Queens and workers show similar rates of DNA damage accumulation with age

In heads of 1-day-old individuals, levels of DNA damage were higher in workers than queens (mean percent damage in comet tail ± s.e.: workers: 20.4 ± 0.6; queens: 17.5 ± 0.9; $n_{workers} = 12$, $n_{queens} = 13$, $\chi^2 = 6.07$, df = 1, $P = 0.014$; Fig. 1a). Levels of DNA damage increased significantly with age in both queens ($n_{1\text{-day}} = 13$, $n_{1\text{-year}} = 12$, $\chi^2 = 26.88$, df = 1, $P < 0.0001$) and workers ($n_{1\text{-day}} = 12$, $n_{1\text{-year}} = 12$, $\chi^2 = 14.72$, df = 1, $P = 0.0001$). This increase was greater in queens than workers (interaction between age and caste: $\chi^2 = 9.12$, df = 1, $P = 0.0025$). Levels of damage did not significantly differ between 1-year-old queens and workers, although there was a non-significant trend for queens to have higher levels of damage (workers: 29.0 ± 0.8; queens: 32.6 ± 1.2; $n_{workers} = 12$, $n_{queens} = 13$, $\chi^2 = 2.74$, df = 1, $P = 0.098$; Fig. 1a).

In the legs of 1-day-old individuals, there was no significant difference in the levels of DNA damage between queens and workers (workers: 25.4 ± 1.7; queens: 24.1 ± 1; $n_{workers} = 12$, $n_{queens} = 14$, $\chi^2 = 0.35$, df = 1, $P = 0.56$; Fig. 1b). Levels of DNA damage also increased significantly with age in both queens ($n_{1\text{-day}} = 14$, $n_{1\text{-year}} = 12$, $\chi^2 = 9.12$, df = 1, $P = 0.0025$) and workers ($n_{1\text{-day}} = 12$,

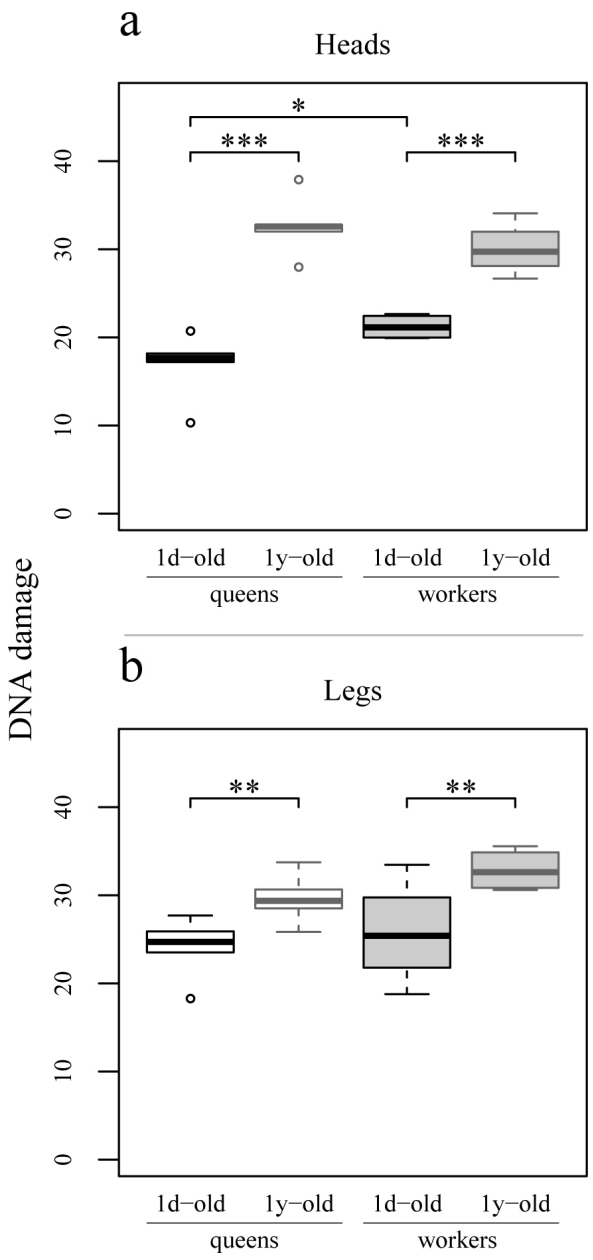

Fig. 1. Levels of DNA damage estimated as percentage of DNA in the tails of the comet assay. In both heads and legs, 1-year-old individuals showed more damage than 1-day-old individuals, but there was no difference in damage levels between 1-year-old queens and 1-year-old workers. Significant differences are shown for comparisons between castes of the same age and between ages of the same caste (* = $P < 0.05$, $P < 0.01$, $P < 0.001$). a) DNA extracted from heads. $n_{1\text{-day-workers}} = 12$, $n_{1\text{-day-queens}} = 13$, $n_{1\text{-year-workers}} = 12$, $n_{1\text{-year-queens}} = 12$. b) DNA extracted from legs. $n_{1\text{-day-workers}} = 12$, $n_{1\text{-day-queens}} = 14$, $n_{1\text{-year-workers}} = 12$, $n_{1\text{-year-queens}} = 12$.

$n_{1\text{-year}} = 12$, $\chi^2 = 8.08$, df = 1, $P = 0.0045$), but there was no significant difference in the rate of increase ($\chi^2 = 0.27$, df = 1, $P = 0.6$). Accordingly, levels of damage did not differ significantly between 1-year-old queens and workers, although there was a non-significant trend towards workers have higher levels of damage (workers: 32.2 ± 1.1; queens: 29.6 ± 0.9; $n_{workers} = 12$, $n_{queens} = 12$, $\chi^2 = 2.95$, df = 1, $P = 0.086$; Fig. 1b).

Levels of DNA damage were overall higher in legs than heads (heads: 24.7 ± 1; legs: 27.7 ± 0.7; $n_{workers} = 49$, $n_{queens} = 50$, $\chi^2 = 13.86$, df = 1, $P = 0.0002$).

3.2. Levels of ubiquitinated protein decrease with age

Levels of ubiquitinated proteins were similar in the heads of 1-day-old queens and 1-day-old workers (ratio of WB to Coomassie density normalised by mean across gel: workers: 0.13 ± 0.04; queens: 0.09 ± 0.02; $n_{workers} = 4$, $n_{queens} = 4$, $\chi^2 = 1.09$, df = 1, $P = 0.3$; Fig. 2a). The levels of ubiquitinated proteins decreased with age in both queens ($n_{1\text{-day}} = 4$, $n_{1\text{-year}} = 4$, $\chi^2 = 11.36$, df = 1, $P = 0.0007$) and workers ($n_{1\text{-day}} = 4$, $n_{1\text{-year}} = 4$, $\chi^2 = 10.18$, df = 1, $P = 0.0014$), and 1-year-old queens had lower levels of ubiquitinated proteins than

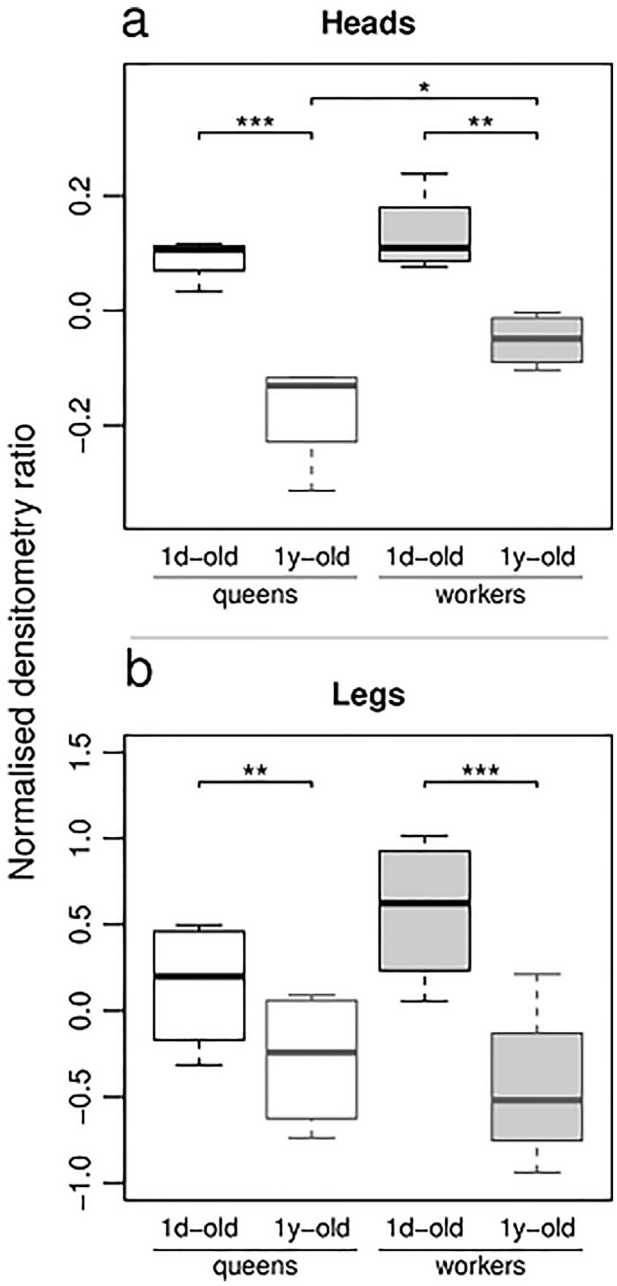

Fig. 2. Levels of ubiquitination in protein extracts. "Densitometry ratio" represents the intensity of fluorescence from the Western Blot divided by the intensity of Coomassie Brilliant Blue on the Coomassie gel. Values are normalised across gels by subtracting the mean densitometry ratio of the gel from each value. In both heads and legs, protein ubiquitination levels were lower in 1-year-old than 1-day-old individuals. Ubiquitination levels were higher in 1-year-old worker head than 1-year-old queen heads. Significant differences are shown for comparisons between castes of the same age and between ages of the same caste (* = $P < 0.05$, $P < 0.01$, $P < 0.001$). Each age/caste combination had four replicates. a) Protein extracted from heads. b) Protein extracted from legs.

1-year-old workers (workers: -0.05 ± 0.02; queens: -0.17 ± 0.05; $n_{workers} = 4$, $n_{queens} = 4$, $\chi^2 = 3.97$, df = 1, $P = 0.046$).

Levels of ubiquitinated proteins were similar in the legs of 1-day-old queens and 1-day-old workers (workers: 0.58 ± 0.22; queens: 0.14 ± 0.19; $n_{workers} = 4$, $n_{queens} = 4$, $\chi^2 = 2.47$, df = 1, $P = 0.12$; Fig. 2b). Levels of ubiquitinated proteins decreased with age in both queens ($n_{1\text{-day}} = 4$, $n_{1\text{-year}} = 4$, $\chi^2 = 10.78$, df = 1, $P = 0.001$) and workers ($n_{1\text{-day}} = 4$, $n_{1\text{-year}} = 4$, $\chi^2 = 11.41$, df = 1, $P = 0.0007$) and there was again no difference in the levels of ubiquitinated proteins between 1-year-old queens and 1-year-old workers (workers: -0.44 ± 0.24; queens: -0.28 ± 0.20; $n_{workers} = 4$, $n_{queens} = 4$, $\chi^2 = 0.31$, df = 1, $P = 0.58$).

4. Discussion

Levels of DNA damage increased with age in both queens and workers. Similar results have been found across a range of taxa (Aamodt, 2009; Behrens et al., 2014; Diem et al., 2002; Giovannelli et al., 2003; Juliet et al., 2005; Kraytsberg et al., 2006; Piperakis et al., 2009; Swain and Subba Rao, 2011; Van Remmen et al., 2003; Vermulst et al., 2007; Wang et al., 2010). Surprisingly, however, we found no evidence that DNA damage increases more rapidly in workers than queens over the first year of their life, despite previous results showing that queens show higher expression of DNA repair genes than workers (Lucas et al., 2016). A possible explanation for this finding is that queens are subjected to higher rates of damage than workers because of increased investment into reproduction, and their increased levels of repair serve to compensate for this. In line with this, the expression of genes linked to metabolism are consistently found to be overexpressed in social insect queens compared to workers (Bonasio et al., 2010, Grozinger et al., 2007, Smith et al., 2008, Lucas et al., under review). This would also explain why differences in DNA repair gene expression between queens and workers are only found in older individuals (Lucas et al., 2016) since queens only begin egg-laying after their mating flight, which occurs when they are around two weeks old.

Another possible explanation is that the increased expression of DNA repair genes in queens results in the reduced accumulation of a different kind of DNA damage, such as oxidised bases. Direct measurement of oxidative damage was not possible as it requires relatively large amounts of biological material, thus we cannot exclude this possibility. However, oxidative damage to DNA can lead to strand breakage (Aust and Eveleigh, 1999), and we would thus expect an excess of oxidised bases to be accompanied by increased strand breaks detectable by the comet assay.

The range of damage levels which we observed (17–33% DNA in comet tail) are relatively high compared to insect studies that used various tissues, where results are typically on the scale of up to 20% (e.g. Augustyniak et al., 2006; Augustyniak et al., 2015; Augustyniak et al., 2014). Nevertheless, our analysis was able to detect changes in DNA damage with age, and thus any biologically meaningful differences in damage in old individuals should be detectable with this method.

Our findings indicate that queens do not achieve their extended lifespan by limiting the accumulation of DNA damage. So far, evidence supporting a link between DNA damage repair and ageing has primarily come from human pro-geroid diseases and interspecific comparisons in mammals (Cortopassi and Wang, 1996; Freitas and de Magalhaes, 2011; Grube and Bürkle, 1992; Lorenzini et al., 2009; Moskalev et al., 2013). The contrasting conclusions between these studies and ours may be explained by the fact that there is only limited cell division in insect somatic tissue (Abernethy, 1998; Rockstein, 1973). While damage to DNA does occur in non-dividing cells, it can also be introduced during replication and the resulting mutations can spread through a cell population by cell division. DNA damage may therefore be especially important in mitotic tissues and it might be that such damage plays an important role in senescence in mammals and other organisms where there is extensive somatic cell replication during adulthood. In non-dividing cells, DNA damage accumulates but its impact remains restricted to the cells in which it occurs and may thus be of little consequence to the longevity of the organism. Our results highlight the fact that the physiology of ageing may vary strongly between taxa. The wear and tear that accompanies senescence is not universal and increases research is needed in non-model organisms to understand the full breadth of biological ageing.

The comet assay is an established technique for investigating DNA damage in the context of ageing in mammals, where it has been used to demonstrate a natural accumulation of DNA damage with age in rats (Giovannelli et al., 2003; Swain and Subba Rao, 2011) and humans (Diem et al., 2002; Piperakis et al., 2009). The comet assay has been relatively recently extended to insects. First reports from 2002 (Bilbao et al., 2002) describe DNA damage in brain ganglia of *Drosophila*, and the method has since been applied to Diptera, Coleptera, Lepidoptera and Orthoptera (Augustyniak et al., 2016). To our knowledge, ours is the first study to extend the use of the comet assay to social insects. Until now, the comet assay has been used only in short-lived insect species (Augustyniak et al., 2016), mechanisms of DNA damage control could be more efficient in long-lived species. A study of DNA damage of long-living insects, including social insects, across their whole lifespan would be of great interest.

Surprisingly, our analysis revealed that protein ubiquitination decreased with age. In contrast, previous studies showed that levels of ubiquitinated proteins increase with age in worms and fruit flies (Liu et al., 2011; Tonoki et al., 2009). Since ubiquitin is a tag for proteins that need to be broken down, a reduction of ubiquitinated protein levels with age may indicate a reduction in the extent to which proteins are being tagged, rather than a reduction in the quantities of damaged proteins. One possible explanation for the decrease in ubiquitinated proteins with age in our study is therefore that our young age group was only one day old. These individuals are emerging from metamorphosis, a state of important physiological transition, and thus stage-specific levels of ubiquitin tagging may override an effect of age. However, in *Drosophila*, an increase in protein ubiquitination with age was detected where the youngest age group was only 2 days old (Tonoki et al., 2009). Furthermore, a study in the honeybee, in which all individuals were already several weeks old, showed no increase in the level of ubiquitinated proteins with age (Tolfsen et al., 2011), suggesting that this lack of increase is not restricted to ants. Overall, results from social insects thus undermine the idea emerging from studies of model organisms that an increase in poly-ubiquitinated damaged proteins is a hallmark of ageing, and show instead that patterns of damaged protein tagging with age vary between taxa.

In conclusion, our study reveals that the previously described difference in expression of molecular repair genes between queens and workers in *L. niger* does not result in differential accumulation of macromolecular damage. A possible explanation for this paradox may be that the elevated expression of repair genes in queens is a necessary response to their increased investment into reproduction, highlighting the importance of the confounding effects that life-history differences can exert on patterns linked to senescence.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.exger.2017.03.008.

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